Infectivity of different Zika virus strains *in vitro*

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Tiivistelmä Referat – Abstract

Zikavirus on *Flavivirus*-sukuun kuuluva taudinaiheuttaja, joka leviää pääasiassa *Aedes*-suvun hyttysten välityksellä, mutta myös seksin ja verensiirtojen kautta, sekä äidistä sikiöön. Ennen laajoja epidemioita Mikronesiassa 2007 ja Ranskan Polynesiassa 2013, zikavirus on aiheuttanut vain yksittäisiä tautitapauksia Afrikassa ja Aasiassa. Viimeisin ja merkittävin epidemia puhkesi Brasiliassa 2015, josta se on levinnyt laajalti Etelä- ja Keski-Amerikkaan.

Zikavirus aiheuttaa tyypillisesti oireettoman tai lievän, itsestään ohimenevän kuumetaudin, johon voi liittyä esimerkiksi ihottumaa, päänsärkyä, nivelkipuja ja silmätulehduksia. Viimeisimmän epidemian aikana suurta huomioita on kuitenkin herättänyt huomattavasti lisääntynyt vastasyntyneiden mikrokefaliatapausten määrä. Zikaviruksen onkin osoitettu aiheuttavan mikrokefaliaa ja lisäksi sen yhteydestä kasvaneeseen Guillain-Barré-oireyhtymän esiintyvyyteen epidemia-alueilla on saatu todisteita. Syyt yhtäkkisten laajojen epidemioiden ja lisääntyvien zikavirusinfektioon liittyvien neurologisten oireyhtymien taustalla ovat vielä epäselviä.

Tässä tutkimuksessa vertasimme kolmen aasialaista ja yhden afrikkalaista tyyppiä olevan viruskannan infektio- ja replikaatiokykyä kymmenessä ihmis- ja kolmessa hyttyssolulinjassa. Havaitsimme, että viimeisimmän epidemian aikana sikiön aivoista eristetty viruskanta FB-GWUH-2016 infektoi useita viruksen patogeneesin kannalta olennaisia solulinjoja tehokkaammin kuin muut Aasian linjaa olevat Martiniquessa ja Ranskan Polynesiassa oireisten potilaiden seerumista eristetyt kannat. Tämän taustalla voisivat olla sopeutumat zikaviruksen genomissa, jotka olisivat muuttaneet viruksen patogeneettisiä ominaisuuksia, esimerkiksi suosien keskushermostohakuisuutta ja -tartuttavuutta. Näiden geneettisten muutosten funktion selvittäminen edellyttää lisätutkimuksia, joihin tämä tutkimus luo hyvän pohjan. (197)

Avainsanat – Nyckelord – Keywords

Zika Virus, Zika Virus Infection, Microcephaly,

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Tiivistelmä – Referat – Abstract

Zika virus (ZIKV) is an arthropod-borne virus of the genus *Flavivirus*. It is transmitted by *Aedes* mosquitos but also sexually, via blood transfusions and perinatally from mother to foetus. Before the outbreaks in Yap Island in Micronesia (2007) and French Polynesia (2013), ZIKV has caused only sporadic human infections in Africa and Asia. The latest and most remarkable epidemic started in Brazil in 2015 and it has later spread to many countries of South and Central America.

Zika virus infection is typically asymptomatic or causes a self-limiting febrile illness with rash, headache, arthralgia and conjunctivitis. During the latest epidemic, the increasing numbers of newborns with microcephaly has attained wide concern. Later, the causality between Zika virus infection and microcephaly has been proven. The virus has also been connected with other neurological disorders, such as Guillain Barré syndrome. Causes of the abrupt epidemics and the increasing amount of neurological symptoms associated with ZIKV infections are still unclear.

In this study, we compared the infectivity of three Asian and one African ZIKV strain in 10 human and three mosquito cell lines. We noticed that a recent ZIKV isolate from foetal brain infected several cell lines more efficiently than two other Asian strains from symptomatic febrile patients from Martinique and French Polynesia. The three viruses of Asian lineage are closely related, but contain specific mutations in their genome. Further studies are warranted to specifically determine the mutations resulting in the increased infectivity of the foetal brain isolate. (244)

Avainsanat – Nyckelord – Keywords

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1 Introduction

Zika virus (ZIKV) has attracted wide publicity during the past few years by causing an epidemic in the South and Central America in 2015-2016. Hundreds of thousands of infections were reported, with an alarmingly increasing number of newborns with microcephaly born to mothers with recent ZIKV infection. The causality between congenital ZIKV infection and microcephaly has later been proven. ZIKV infection has also been connected with Guillain-Barré syndrome, acute myelitis and meningoencephalitis (1-3).

Previously, the virus has caused asymptomatic infections or only mild, self-limiting symptoms like rash, headache, fever and arthralgia. ZIKV is an arthropod-borne virus (genus *Flavivirus*, family *Flaviviridae*) transmitted by mosquitos of *Aedes* genus, *Aedes aegypti* being its main vector. The diagnosis is often missed or mixed with infections by other species of the genus *Flavivirus* causing similar symptoms.

After its discovery in 1947, ZIKV has caused only sporadic infections in Africa and Asia until the first outbreak in Yap state in Micronesia 2007 (4). Second outbreak occurred in French Polynesia in 2013 causing over 19 000 infections (5). After that, the virus caused only a few human cases until the early 2015 when increasing number of ZIKV infections were reported in northeast Brazil. Later on, the virus spread to all federal units of Brazil and numerous cases were diagnosed in different South and Central American and Caribbean countries. Due to the exceptionally high number of microcephaly cases associated with ZIKV infections, the Brazilian Ministry of Health proclaimed a public health emergency in November 2015 and in February 2016, WHO declared the ZIKV

outbreak a Public Health Emergency of International Concern (PHEIC) (6). It has also been shown that the virus can be transmitted sexually and via blood transfusions. As far as the congenital abnormalities are concerned, the perinatal transmission is of importance (7,8).

During the latest outbreak, when the connection between neurological abnormalities and ZIKV infection has strengthened, the attitude towards the virus has completely changed. As there is no approved treatment or vaccine against ZIKV yet, the importance of prevention has to be emphasized. Actions to control mosquito populations and to prevent mosquito bites are applied in areas of autochthonous infections. It is important to raise awareness of sexual transmission and especially to prevent infections of pregnant mothers worldwide. The most intriguing question is, however, why this virus suddenly caused such vast epidemics and what is the reason for the increasing number of microcephaly and other neurological syndromes associated with ZIKV infections. Is it only a consequence of the changing environment or is there an enhancement in the infectivity and pathogenicity of the virus? Musso *et al.* estimated that the most likely reason may be new genetic adaptations in the ZIKV genome leading to higher virulence, other reasons being low immunity against ZIKV in the Americas, globalization, urbanization, world population growth, poor vector control and changes in vector competence (9).

In this study, we investigated the differences in replication properties and infectivity of different ZIKV strains. We compared the viral strain FB-GWUH, recently isolated in our laboratory from brain tissue of a foetus from a mother with ZIKV infection, to two epidemic strains of the same Asian lineage, MRS OPY and H/PF, from Martinique and

French Polynesia, respectively (10). We also included the prototypic strain of the African lineage, MR766, in the study. We measured the replication efficiency and infectious virus production in 10 human and 3 mosquito cell lines 3 days post infection. We also investigated whether there is a difference in the antiviral response in the different cell lines by measuring the antiviral MxA mRNA.

2 Review of the literature

2.1 Virus structure and genetics

Zika virus is a member of the genus *Flavivirus,* family *Flaviviridae,* together with 50 viruses including dengue, Japanese encephalitis, tick-borne encephalitis, West Nile and yellow fever viruses (hereafter referred to as flaviviruses). Most flaviviruses are arthropod-borne viruses and they can be divided into four groups based on their vectors and ecological and phylogenetic features; two mosquito-borne, tick-borne and non-vectored group. There are also some mosquito-borne viruses that are insect-specific and seem not to infect vertebrate cells at all (11,12). According to phylogenetic studies of tick-borne flaviviruses, the tick-borne flavivirus appeared over 16 000 years ago, and the common ancestor of all flaviviruses probably has existed thousands of years longer (11). The flavivirus genome is composed of a single-stranded positive-sense RNA of approximately 11 kilobases in length, which encodes three structural and seven nonstructural proteins in one open reading frame (Fig 1.). The polyprotein is cleaved into structural proteins C (capsid), prM (precursor of membrane) and E (envelope), and nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (13). These proteins have quite the same structure in all flaviviruses except for minor differences for

example in glycosylation sites (14). The flaviviruses go through three developmental states during their lifecycle: immature, mature and fusogenic, of which it is infectious only in the mature state. The most important part of the maturation takes place in the Golgi apparatus where prM is cleaved into M protein and critical conformational changes of its surface glycoproteins are performed (15). Structural proteins C, M and E make up the virus particle. The icosahedral capsid encloses the genome and is surrounded by a lipid membrane, where glycoproteins M and E heterodimers are anchored (Fig.2). The E protein consists of three domains, typical of flaviviruses. It acts in virus entry and is found the target of neutralizing antibodies (16).

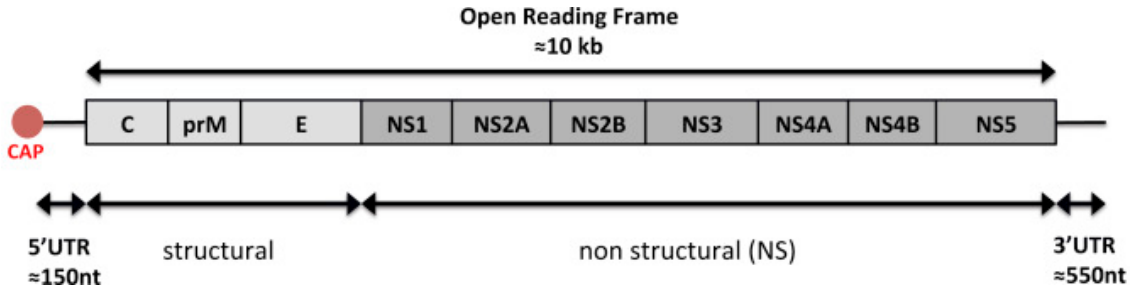

Figure 1. ZIKV genome structure. Reproduced with permission from Hamel *et al Microbes and Infection, 2016* (17).

The nonstructural proteins function in replication, antagonism of the host cell immune response and virion assembly (15,16). NS1 is the only glycosylated non-structural protein. It acts in ZIKV replication and interacts with the host innate and adaptive immune system. Because it is also secreted in the host extracellular space, it can be used as a flavivirus infection marker. NS3 has helicase activity so it unwinds the RNA during virus replication. It is also involved in polyprotein processing as a serine protease with NS2B as a cofactor. NS5 is the largest non-structural protein containing an N-terminal

methyltransferase and an RNA-dependent RNA polymerase domain. The methyltransferase inserts a cap structure into the 5`-end of the mRNA, which protects the molecule from host immune system and helps in translation. The RNA-dependent RNA polymerase is the enzyme, which adds single-nucleotide triphosphates into the upcoming RNA molecule during RNA synthesis. The structure and function of NS2A, NS2B, NS4A and NS4B have been associated in the antagonism of innate immune responses and the assembly of the virus replication machinery (18-20).

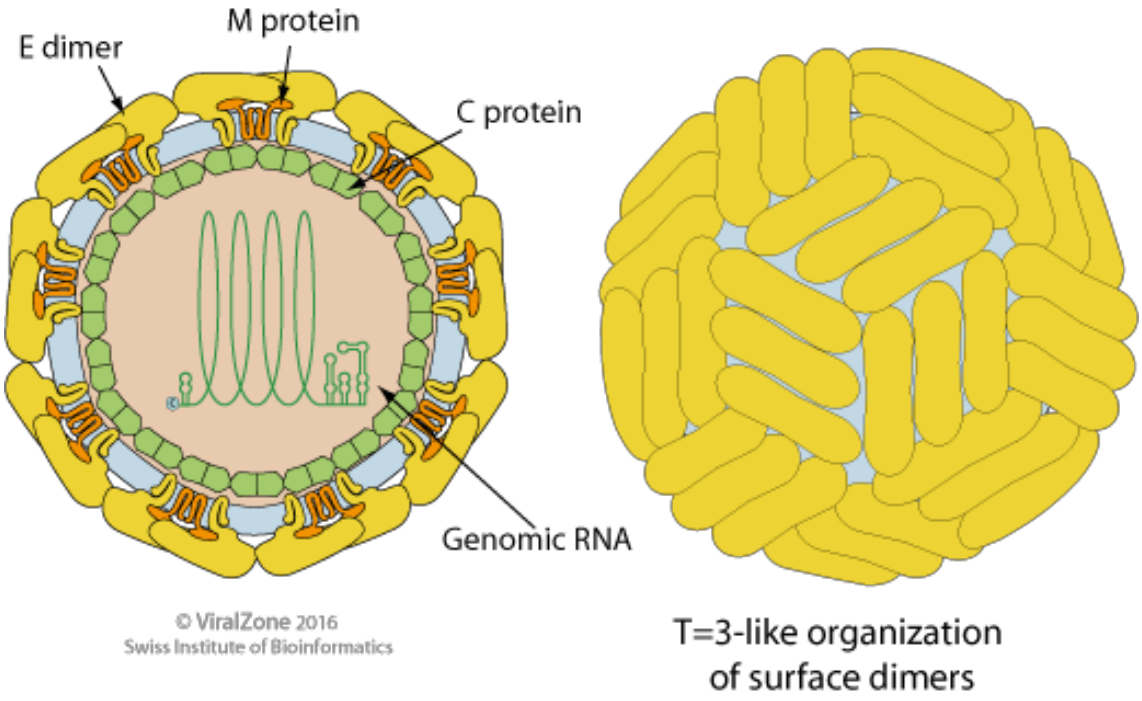

Figure 2. Zika virus structure, reproduced with permission from Swiss Institute of Bioinformatics, ViralZone.

There are two main genomic lineages of ZIKV: the African and the Asian (21). The Ugandan strain, MR766 seem to be diverged first and then the other two African lineages (Nigeria and Senegal). The genetic variation is biggest between the African and Asian lineages and smaller among each lineage. Most of the variation is deletions of

glycosylation sites (21). Genomic sequences of ZIKV strains from South and Central America are closer to Asian than African lineages, but they seem to be closer to French Polynesian strain than Southeast Asian strain. Therefore, the American strains are not likely to be direct descendants of the Southeast Asian strains (22).

2.2 Epidemiology

Despite the massive attention given to Zika virus recently, it is not actually a new pathogen in the environment. Zika virus was first isolated from a feverish Rhesus monkey in Zika Forest near Lake Victoria in Uganda 1947 during research of yellow fever. After that it has been isolated from its vector *Aedes africanus* mosquito in 1948 in the same area (23). The virus was known to cause human infections in Uganda and Nigeria; there was a 6,1% seroprevalence in people living near the Zika forest (24,25). More proof of infectivity was attained from studies of an outbreak of jaundice in Nigeria in 1952, when the virus was isolated from serum of a 10-year old girl with fever and headache but no jaundice. Neutralizing antibodies against Zika virus were present in serum of all three patients included in the study (26).

Zika virus antibodies have been detected in some arbovirus epidemiological studies done in different parts of Africa during following decades of its discovery, seropositivity being highest in Nigeria (ad 56%) (27-30). Serological survey in Southeast Asia also revealed antibodies against Zika virus among people living in that part of the world (31,32). The virus was later isolated from *Aedes aegypti* mosquito in Malaysia supporting that Zika virus was causing human infections in Southeast Asia (33). In 1977 and 1978 seven

serologically confirmed cases were reported in Tegolyoso Hospital in Java, Indonesia when febrile patients were tested for alphavirus and flavivirus infections (34).

Taken together, there have been only sporadic documented Zika virus infections since the first isolation to the beginning of the 21st century and none of them outside Africa and Asia. ZIKV infections are probably grossly underdiagnosed because of the typically mild symptoms and similarities to dengue infections (21). However, in 2007 about 75 per cent of the 7 000 inhabitants of Yap islands were infected with Zika virus. Yap state is part of the Federated states of Micronesia which is located in the western Pacific Ocean, northeast of Papua New Guinea (35). From the beginning of April to the end of July 2007 Duffy and colleagues investigated blood samples of 185 suspected Zika virus cases, 49 of which confirmed to be positive and 59 suspected. The infection was confirmed by detection of ZIKV RNA in the serum by reverse transcription polymerase chain reaction (RT-PCR) and by identifying IgM against Zika virus by enzyme-linked immunosorbent assay (ELISA) and plaque-reduction neutralization tests (PRNT). It is thought that the virus has not existed in the area previously because of the sudden accumulation of a lot of cases during short time and a high IgM seroprevalence against ZIKV (35).

After the outbreak in Yap island the second remarkable epidemic took place in French Polynesia, a cluster of islands located in the southern Pacific Ocean in 2013. There were several patients with mild fever, arthralgia, headache and rash, who were negative for dengue virus and later tested for ZIKV virus by RT-PCR. Altogether more than half of the nearly 600 tested serum samples were positive for ZIKV. It was estimated that there were over 19 000 suspected ZIKV infections until the end of 2013 (5). During this

epidemic ZIKV was suspected for the first time to cause Guillain-Barré syndrome in addition to the typical mild symptoms (36). In December 2015, a confirmed autochthonous human case was reported in Martinique, an insular territory in the eastern Caribbean Sea. Until February 2016 over 7 000 clinically suspected cases were announced in Martinique (37).

ZIKV cases have been reported also in other Oceanian countries: Easter Islands, Cook Islands, New Caledonia, Vanuatu and Solomon Islands. The viruses have probably spread from people travelling from French Polynesia to these islands and bitten by local vectors (38-40).

The largest outbreak started in northeast Brazil in early 2015. In the beginning of 2015, eight patients from Bahia and Rio Grande do Norte states, who had fever, rash, arthralgia and conjunctivitis, were diagnosed with ZIKV infection by RT-PCR and sequencing in May 2015 (41). All of them were tested to be DENV and Chikungunya virus (CHIKV) negative. Later on, ZIKV has spread widely in Brazil and cases have been documented in all 27 federal units. According to the estimates of the Brazilian ministry of health there were 440 000 to 1 300 000 cases in 2015 in Brazil (42). Highest incidence of 663/100 000 was reported in Mato Grosso state in western Brazil. Eleven deaths related to ZIKV infections have been reported until the beginning of 2017 (43).

ZIKV has not stayed inside of Brazilian borders but it has taken over many South and Central American and Caribbean countries including Colombia, Bolivia, Mexico and Martinique; over 26 American countries have reported indigenous ZIKV infections

(6,44,45). When it comes to North America and Europe, autochthonous infections are rare (Fig 3.).

According to the report of the European Center for Disease Prevention and Control (4/2017) Texas reported its first confirmed locally acquired case in November 2016 and five additional cases have been diagnosed later. Florida reported two confirmed asymptomatic ZIKV cases in March 2017. In Europe, no vector-borne transmissions have been detected, but six European countries have reported 20 sexual transmissions of ZIKV altogether. During 2016 the infections in the Americas and Caribbean decreased and the epidemic ended. In November 2016 WHO proclaimed that the ZIKV epidemic was no longer considered as a PHEIC (46).

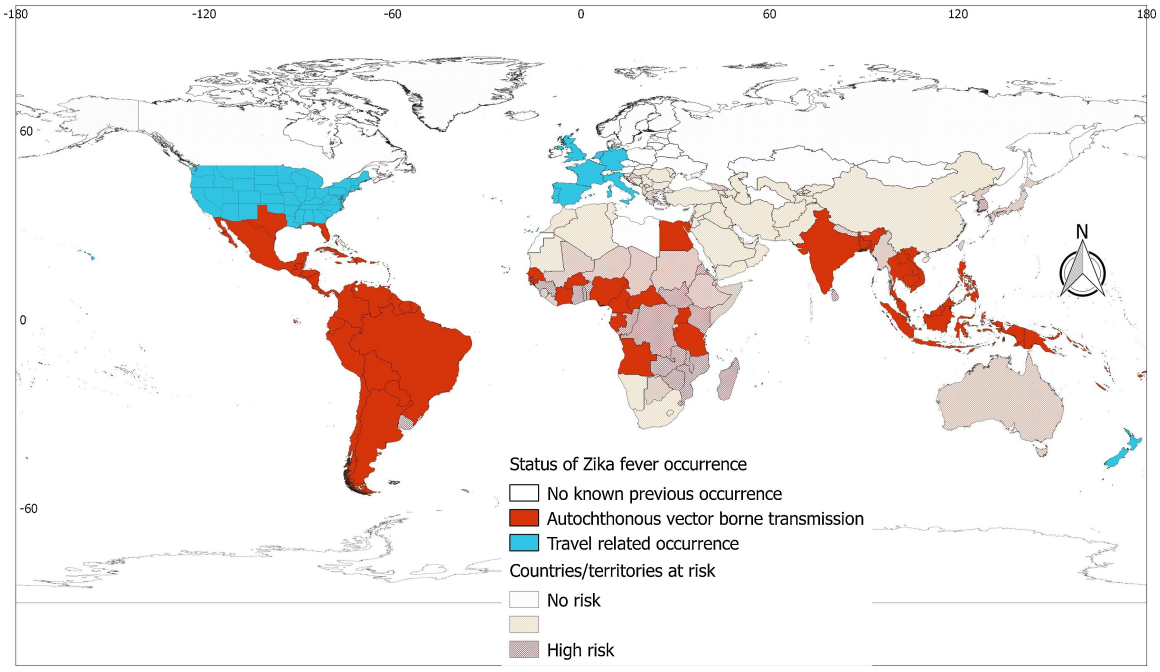

Figure 3. Global occurrence of Zika fever in 2017. Reproduced with permission from Leta *et al* *IJID 2018* (47)

2.3 Transmission

Zika virus typically circulates between mosquitos and non-human primates in sylvatic cycles in rural areas, causing only occasional human infections. In order to survive in urban areas with no non-human primates the virus has adapted to a mosquito-human-mosquito transmission cycle (9). Primates are thought to be the main host for ZIKV, but ZIKV antibodies have been detected in other animals like elephants, lions, hippos and goats (21,48). The mosquitos responsible for ZIKV transmission are of *Aedes* genus (*Ae*.). *Ae. africanus* was the first species from which ZIKV was isolated in 1948 but later it has been extracted from *Ae. aegypti, Ae. apicoargenteus, Ae. luticocephalus, Ae. furcifer* and *Ae. vittatus* in Africa. *Ae. aegypti* is considered the main vector of ZIKV and is the only mosquito species from which it has been isolated outside Africa. *Ae. hensilii* was thought to be the most important vector during the Yap outbreak 2007 but the virus could not be extracted from any mosquito species there (33,35,49). *Ae. albopictus*, a mosquito species widely spread from Asia to Europe, America, Africa, Australia and the Pacific Island, has been showed to be a potential vector of ZIKV (50). *Culex* mosquitos, the vectors of for example West Nile virus and Japanese encephalitis virus, have not been found to be competent vectors for ZIKV (51-53).

The virus is contained within the mosquito for an extrinsic incubation time of approximately 10 days and is then secreted into the saliva (49). When the infected mosquito bites a human, the virus is transferred into the skin through the inoculation site. The virus typically has an intrinsic incubation time of 4-5 days during which it likely replicates in skin dendrites and translocates via the lymphatic system into the blood

stream. From blood, the virus can be transferred into another vector during blood feeding (54).

Zika virus can also be transmitted sexually. Several cases of women who have symptoms typical of ZIKV infection without staying recently in endemic areas have been reported (55,56). Their sexual partners have been travelling in areas of known local Zika virus transmission and have had similar symptoms within two weeks before women. Some male patients having ZIKV infection have been noted to have hematospermia and symptoms of prostatitis (perineal pain and dysuria). High loads of ZIKV RNA have been extracted from semen and replicative virus particles have been detected in cell cultures (57,58). According to a case report, it seems that ZIKV may be transmitted through anal sex as well (59). ZIKV RNA has also been extracted from saliva but transmission through it, and not semen, during oral sex has not been shown (7).

Given the lately found birth defects associated with ZIKV infections, the perinatal way of transmission is essential for the pathogenesis and prevention of Zika virus. First evidence of perinatal transmission emerged during the French Polynesian epidemic 2013-2014 when newborns of two mothers having ZIKV symptoms, were tested positive for ZIKV (60). It could not be confirmed, whether the infection spread transplacentally or during delivery. More proof of perinatal transmission has been documented constantly when cases of congenital foetal abnormalities associated with maternal ZIKV infection have been reported (2,45,61). The virus has also been isolated from the foetal brain tissue, which for its part, supports the causality (10). There is an examination in which foetuses of ZIKV infected pregnant SJL mice showed intrauterine growth restriction, and

microcephaly supporting the hypothesis of vertical transmission (62). ZIKV RNA has been detected in breast-milk but it could not have been proven that the virus can be transmitted by it (63).

ZIKV can also be transmitted through blood transfusions. Motta and colleagues reported a case of a platelet donor who developed rash, retro-orbital pain and pain in both knees five days after donation and later tested positive for ZIKV RNA. The two recipients of his platelets remained asymptomatic, but their serum tested positive for ZIKV in RT-PCR (8). Pre-transfusion screening of donors for ZIKV RNA by nucleic acid testing (NAT) has proven to be a possibly effective method in controlling infections via blood transfusions (64).

2.4 Clinical picture

ZIKV infection typically causes an asymptomatic (in approximately 80% of cases) or mild, self-limiting febrile disease. The symptoms are not specific to ZIKV infection and they can easily be regarded as features of dengue or chikungunya infection that circulate in the same geographical areas. The rash is, however, more common in ZIKV than in other arthropod-borne virus infections (65). Congenital ZIKV infection has been shown to cause microcephaly (2). There is also some evidence of connection with other craniofacial malformations (66). In adults, the virus is suspected to be associated with Guillain-Barré syndrome and possibly myelitis and meningoencephalitis (3,67).

2.4.1 Febrile illness

Referring to a review of case reports from the outbreaks in Yap island and French Polynesia, the most common symptoms are maculopapular rash typically in palms and soles, fatigue or lethargy, mild fever, arthralgia especially in knees, hands and feet, conjunctivitis and headache. Retro-orbital pain, vomiting, lymphadenopathy and hematospermia have also been reported (9). The blood count is usually normal or minor leukopenia or thrombocytopenia is detected. C-reactive protein is typically normal or only slightly elevated. The incubation period between the infection and the beginning of the symptoms is 3-12 days and the symptoms usually persist about one week (9,65).

2.4.2 Guillain-Barré syndrome

During the French Polynesian outbreak there was an abrupt increase in the incidence of Guillain-Barré syndrome (GBS) (3). GBS is an autoimmune disease causing typically bilateral weakness, pain and paresthesia of limbs, usually beginning in the distal parts. It often develops approximately three weeks after infection, for example with *Campylobacter jejuni*, Cytomegalovirus or Epstein-Barr virus, when antibodies against the pathogen cross-reacts with myelin of peripheral neurons (68). Between November 2013 and February 2014, 42 GBS cases were reported from French Polynesia, which is a huge number of patients compared to the average of 5 cases per year in the four previous years (3). Most of the patients had typical mild ZIKV symptoms approximately six days before the appearance of neurological symptoms and almost all of the cases had anti-ZIKV IgM antibodies in their serum indicative of an acute ZIKV infection.

More support to the spatial and temporal correlation of ZIKV infection and GBS have come from the areas of the third outbreak when many countries in Latin America, including Brazil and Colombia, and the Caribbean have documented exceptionally large numbers of cases (69). In Colombia, there were 270 confirmed cases of GBS associated with ZIKV infection between October 2015 and March 2016 and it is estimated that during the ZIKV outbreak the frequency of GBS was 90 cases per month. The frequency was approximately 20 per month before the outbreak (70). These epidemiological data and temporal correlations, in addition to the neurotropism of the Zika virus, strongly support the causality between ZIKV infection and GBS but more data of the pathogenesis is needed.

2.4.3 Acute myelitis and meningoencephalitis

There may also be correlation between ZIKV infection and acute myelitis. One report describes a 15-year old girl with left-sided paresthesia, low-back pain and dysuria (71). MRI of the spinal cord showing features of myelitis, and her serum, urine and cerebrospinal fluid (CSF) samples tested positive in ZIKV RT-PCR with high loads of ZIKV RNA. There are also reports of patients with clinical picture of meningitis or meningoencephalitis whose CSF tested positive for ZIKV RNA (72,73)

2.4.4 Microcephaly

Microcephaly is usually defined as an occipito-frontal circumference under 2 standard deviations (SD) of the mean value for age and sex, being 31,9 **cm** for boys and 31,5 cm

for girls at 37 weeks of gestation, according to standards of World Health Organization. A head circumference lower than 3 SDs is considered severe microcephaly. Microcephaly can be primary or secondary if it is seen already at birth or later postnatally. Although most neurons have been generated until the 21st week of gestation, the brain development still proceeds when new dendritic connections are formed and myelination process continues. The most frequent etiologies of microcephaly are genetic anomalies and brain injuries. Congenital infections and teratogenic substances are more rare causes, but the prognosis of microcephaly caused by infections seems to be worse (67,74). Clinical symptoms more common among children with microcephaly are, for example, cerebral palsies, epilepsy and mental retardation (75). Cranial abnormalities include predominant occipital protuberance, redundant scalp and craniosynostosis (66,67).

In May 2016, Rasmussen and colleagues reviewed the data concerning the causality of ZIKV infection and microcephaly. By using the Shephard's and the Bradford Hill criteria as a framework they concluded that there is a causal link between prenatal ZIKV infection and brain abnormalities including microcephaly. Shephard's criteria compose of 7 terms, which are generally used in the evaluation of potential teratogens. Criteria 1-4 are considered most relevant and usually if criteria 1 to 3 or 1, 3 and 4 are met, the causality is considered to be proven (2). With ZIKV infection, criteria 1,3,4 are fulfilled. The first criterion is about the critical timing of the exposure to the teratogen. With ZIKV infection the first trimester and early second trimester seem to be critical time for the development of severe microcephaly, later infection possibly leading to poor overall intrauterine growth or foetal death. The third criterion states that there should be a precise description of typical abnormalities and clinical picture associated with the teratogen. The fourth

criterion is met, if there is an association between a rare exposure and rare defect. Microcephaly is a rare abnormality, incidence being approximately 6 per 10 000 live births in the USA and because there are case reports of people travelling to endemic areas from countries of no ZIKV infections, it can be concluded that in these cases, ZIKV has been a rare exposure leading to a rare defect (2).

From the beginning of the Brazilian outbreak, increasing numbers of babies born with microcephaly were reported (76,77). For example, in the Pernambuco state located in the north-eastern Brazil, the number of cases rose from 140 to 700 in less than a month, at the same time with the ZIKV outbreak. Usually, approximately 150-200 cases are reported annually in the whole country. Almost 3 000 cases had been reported by January 2016 (9). In retrospective studies, an exceptionally high amount of microcephaly has been observed in French Polynesia during the ZIKV outbreak 2013-2014: 17 cases compared to an average of one annual case (78). In late 2016, Brasil and colleagues published a cohort study in which they showed the higher rate of abnormal clinical and imaging findings in the babies born to ZIKV-positive (40%) than the ZIKV-negative mothers (5%) (79). The foetal mortality was similar in ZIKV-infected and non-infected groups.

Infectious ZIKV has successfully been isolated from foetal brain tissue. There are two studies of pregnant women who had stayed in the areas with high incidence of ZIKV infections (10,45). The women suffered typical symptoms of ZIKV infection. Both of the pregnancies were terminated because of abnormal ultrasonographic findings including growth retardation, calcifications and atrophy of the cerebral cortex. The imaging

findings were confirmed on autopsy. Similar dense virus-like particles were detected in electron microscopy in both studies (10).

2.4.5 Other congenital abnormalities

In addition to microcephaly, ZIKV has later shown to be associated with a spectrum of neurological abnormalities and defects in other organs. This group of symptoms and defects is called the congenital Zika syndrome (CZS). Typical neurological findings are small brain size, gyrus pattern simplification or even agyria, asymmetric brain lobes, dilatation of the ventricles, malformation of the corpus callosum, enlargement of the extra axial spaces and calcifications (67). Patterns of severe retardation of brain development such as lissencephaly and holoprosencephaly have also been reported (66) There is also a case report of a foetal demise at 32th weeks of gestation associated with ZIKV infection (80). The foetus presented hydrops fetalis and hydraencephaly and samples from tissues of central nervous system and amniotic fluid tested positive in ZIKV RT-PCR.

Hypertelorism, short nose and flat midface are facial features found to be associated with ZIKV infection in sporadic cases but further studies are needed to ensure the relationship between them (66). Concerning the musculoskeletal system arthrogryptosis and single joint contractures, scoliosis and hip dislocation have been reported (67,81). Ocular findings like macular and optic nerve aberrations have been diagnosed in prenatally infected foetuses (82). There is also some evidence of pulmonary hypoplasia and intra-alveolar haemorrhage, as well as genitourinary tract defects (81).

2.5 Pathogenesis

Considering the symptoms and syndromes ZIKV causes it is crucial to understand the route of the virus from the mosquito into the skin cells and bloodstream and further into the nervous system. In pregnant women the viral entry across the placenta to the foetal central nervous system is under great interest.

2.5.1 Skin cells

ZIKV has been shown to be able to replicate in human skin fibroblasts, keratinocytes and immature dendritic cells. DC-SIGN-, Tyro3-, especially TAM-receptor AXL and to a lesser extent TIM-1- receptor have shown to be important in ZIKV entry (83). Zika virus infection of the skin cells induces a host response: the expression of pattern recognition receptors (PRRs), like TLR3, RIG-I or MDA5, is increased in order to recognize pathogen associated molecular pattern (PAMPs), e.g. double-stranded RNA (dsRNA) (83). The virus particles replicate mainly in the cell cytoplasm, but because ZIKV antigens have also been detected in Vero cell nuclei, nuclear replication is also possible (9). After replication, the progeny virus particles move into regional lymph nodes and into the blood stream (54).

2.5.2 Nervous system

The neuropathogenesis of the virus is not yet completely understood. ZIKV has been shown to infect astrocytes and neural stem cells (NSC) derived from induced pluripotent stem cells (iPSCs) (84). The virus is also able cause infection and induce cell death in human neural progenitor cells (hNPC) (85). The ability to infect these cells of the nervous

system supports the neurotropism of the virus. Degeneration of neurons and glia cells has been detected and ZIKV has been shown to elicit neutrophil invasion in the hippocampus and meninges (86). Necrosis of the pyriform cells, astrocyte hypertrophy and vascular dilatation are further abnormalities found in hippocampus of mouse models (87).

Neurospheres and cerebral organoids have been constructed to better mimic the structure of the neural tissue. Cerebral organoids are three-dimensional models made of embryonic and human pluripotent stem cells resembling the foetal brain structure during the first trimester of gestation. In addition to cell death, reduction of cortical thickness and numbers of cells positive for TBR-1, PAX-6 and CTIP-2, proteins important in brain development, have been detected in cerebral organoids after ZIKV infection (62). Neurospheres (cell cultures containg free-floating neural progenitor cells growing in suspension) showed extensive cell death, morphological abnormalities and reduced size of the NPCs after ZIKV infection (62).

One mechanism behind the dysregulation of neurogenesis, attenuation of growth of the organoids and enhanced apoptosis seems to be the upregulation of Toll-like receptor 3 (TLR3) by ZIKV infection. When using a specific TLR3 inhibitor the growth dysregulation and cell death are remarkably milder after ZIKV inoculation (88). ZIKV has been shown to perturb centrosomal structures and the formation of the plane of mitotic division in NPCs in organoids leading to depletion of NPCs and attenuation of neurogenesis (89).

2.5.3 Placenta and foetus

There is supporting evidence of direct transplacental transmission of the virus if the mother is infected during the first trimester of the pregnancy (66). The TAM- receptors AXL and Tyro3 are widely expressed in trophoblasts and dendritic cells of the placenta providing a possible route of entry for the virus (90). There are several hypotheses concerning the transmission of the virus into the foetus.

One mechanism for ZIKV to get transferred across placental barrier could be the usage of exosomes, vesicles into which the virus would be packaged in the trophoblasts and then transported into the foetus (91). Another route across placenta could be through the system that normally transmits maternal antibodies into the embryo. Some studies show that the transport of antibody-ZIKV complexes via Fc receptors of endothelium of the placental villi and cells of the gut mucosa of the foetus seems to be a favourable and convincing hypothesis, however, there is problem with the timing. The transport of immunoglobulins begins at week 16, peaking as late as 4 weeks before delivery, whereas teratogenicity of ZIKV is highest during the first trimester (90).

ZIKV could also cause neural defects and organ damage indirectly by infecting the placenta. This is suggested if ZIKV infection happens during second or third trimester (90). Infection of the placenta could cause a general immune reaction and inflammatory cytokines would activate microglia, which would end up in disruption of neurogenesis.

Placental invasion of ZIKV might also reduce the production of molecules essential for neurological development or increase the transcription of genes causing microcephaly

(91). However, there is only minor evidence of preterm birth or low birth weight caused by ZIKV infection, which are normally associated with placental dysfunction.

ZIKV has been extracted from placental cells and the virus has been shown to replicate especially in Hofbauer cells, the primary placental macrophages (90). These maternal cells have also been suspected to establish another route of transmission in which these migratory cells could carry the virus across the placenta. Other maternal cells have also been detected in lymph nodes of foetuses during second and third trimester, suggesting transplacental movement (92).

How the virus enters the foetal brain and causes neurological symptoms and congenital neural defects, remains an open question. The histopathological picture of ZIKV infected foetal brain tissue has shown to be quite similar in all reported cases: degeneration of neurons and glial cells, necrosis, gliosis, microglial nodules and microcalcifications (66,81,93). The above mentioned TAM- receptors AXL and Tyro3 together with DC-SIGN and TIM-1 have also been found on the surface of many cells of the central nervous system (90).

2.6 Diagnostics

As the clinical picture of ZIKV infection is often asymptomatic and very similar to other flavivirus infections, reaching the correct diagnosis is more challenging. ZIKV diagnostics are mostly based on serology and molecular techniques.

ZIKV-specific IgM appears in blood within a couple of days after the onset of symptoms and can be detected for up to three months. IgG levels rise later and can last up to months or years. Detection of ZIKV-specific IgM by enzyme-linked immunosorbent assay (ELISA) is one method to diagnose an acute ZIKV infection. However, it is not widely available and there is significant cross-reactivity to other flaviviruses, especially to dengue virus. Pan American Health Organization (PAHO) recommends to repeat the antibody testing after two weeks and if there is an increase in the antibody titer, acute infection is probable (54,94,95). Plaque reduction neutralization test (PRNT) can be used to improve the specificity of ELISA. The cross reactivity is highest among people with previous flavivirus infection. According to serological surveys in Yap Island during the epidemic in 2007, there is some limited cross reactivity in PRNT even in samples of patients without heterogenous flavivirus IgG (4,94).

Molecular detection of ZIKV RNA can be done by conventional or real-time RT-PCR from blood, serum, urine or saliva samples. The PCR method seems to be effective in diagnostics within one week from the onset of symptoms. Viral loads in blood are usually quite low, which reduces the sensitivity of RT-PCR made in blood or serum samples. ZIKV RNA loads have found to be higher and last longer in urine. Urine samples seem to be positive for ZIKV for over 10 days (96). There is a report of a patient whose urine but not serum was positive for ZIKV RNA in RT-PCR about 7 days after onset of the symptoms (97). ZIKV RNA can also be detected in saliva and there is some evidence that RT-PCR is more sensitive in saliva than in serum but it is not widely used because it does not widen the diagnostic window of ZIKV infection. High viral loads have also been detected in semen and breast milk but they are not part of the basic diagnostic protocol

(9). The viremia was found to last longer in a pregnant women whose foetus was infected. A serum sample of the mother was positive for ZIKV RNA in RT-PCR 10 weeks after infection. It is thought that RT-PCR could possibly be used in diagnosing foetal infections in pregnant women(10,98).

However, there are not well studied and effective ways to diagnose foetal infections in practice, but RT-PCR of the amniotic fluid and cord blood at the time of delivery have been used. Ultrasonography and MRI can be used in screening microcephaly and other foetal brain abnormalities. WHO recommends ultrasonography examination between 18th and 20th week for detection of early foetal anomalies. The screening should be repeated at about 30th week. The negative predictive value of ultrasound in diagnosing microcephaly is quite high but the sensitivity is variable and depends largely on technical factors and patient characteristics (10,98,99).

Serological and molecular methods are not exclusive but they are both used and the appropriate approach is chosen based on how many days have gone from the beginning of symptoms. It should also be considered if the person has had previous flavivirus infection, or lives in flavivirus endemic area. According to PAHO guidelines the ZIKV or flavivirus PCR is recommended if the symptoms have lasted less than five days. On the other hand, if more than seven days have gone, IgM serology and PRNT are the most suitable tools. It may be sensible to do both if the time interval is uncertain or between 5 and 7 days. RT-PCR should be done to residents of endemic areas and to people who have had previous flavivirus infection because of the cross-reactivity in the serological tests (9).

2.7 Prevention and treatment

There is no approved anti-viral therapy against Zika virus and the treatment is supportive and symptomatic. Bed rest, fluid replacement and acetaminophen as an antipyretic and analgesic are the basis of the treatment. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are not recommended before dengue infection is excluded because they can cause dangerous bleeding in dengue patients (54,94,100).

The urge to find an effective drug against ZIKV is enormous and increasing number of studies concerning treatment of ZIKV infection are being published since the outbreak in the Americas. In those studies either already existing FDA-approved drugs or completely new agents targeted to specific proteins important in infection or replication cycle of ZIKV are tested in *in vitro* experiments if they can inhibit ZIKV infection. Medicines having anti-viral activity against dengue virus might also be effective in treating ZIKV infections. Passive vaccination and treatment with human monoclonal antibodies being studied with dengue and West Nile virus should also be considered with ZIKV(101).

Non-structural proteins NS5 and NS3 are considered possible candidate as targets of drug design and different substances have been tested how they bind and possibly inhibit molecules essential in replication of the viral genome and protection against host cell immunity (102,103). Some of the already approved drugs have shown inhibitory potential in *in vitro* studies against ZIKV. An anti-parasitic drug suramin may inhibit viral entry and replication and it also interferes with NS3 helicase (104). Bromocriptine has been shown to possess anti-ZIKV activity by reducing the protease activity of NS2B-NS3 (105). Obatoclax, saliphenylhalamide and gemcitabine, drugs previously shown to inhibit

influenza infection, have been also shown to be effective against ZIKV infection *in vitro* (106). Sofosbuvir, a drug against hepatitis C virus, antimalarial drug chloroquine and adenosine triphosphate analogs can also impede ZIKV infection *in vitro* (107-109).

Despite these positive *in vitro* findings, the functional treatment for ZIKV is still to come and a lot of further research is needed. To develop a drug usable in clinical practice, many challenges are still to be resolved; the time of administration with respect to infection and onset of symptoms and whether the drug causes side effects. The drug should also be able to cross blood-brain barrier and placenta and be functional in the nervous system. Possibly evolving resistance of viruses may become a problem in one-drug therapy. The main challenge, however, is to find a medicine both effective and suitable for pregnant women and their foetuses and infants (101,110).

With the lack of an effective therapy to ZIKV infection, it is critical to emphasize the role of prevention. The main means of prevention are vector control, protection against mosquito bites, inhibition of sexual transmission and vaccine development. To limit mosquito populations in endemic areas, it has been noticed to be beneficial to reduce breeding areas and habitats of the larvae, like water containers and used car tires, where rainwater can accumulate. Larvicides are also widely used. However, it has been noticed that only a few sources are enough to maintain the local mosquito population high enough and it might be impossible to eliminate all. Natural predators of the vectors and mosquito traps could also be more efficiently used in the future. Novel technologies to fight against vector-borne infections are being developed. Use of genetically modified male mosquitos and release of Wolbachia bacteria infected *Aedes aegypti* in the environment are two

possible methods. Genetically modified male mosquitos have a dominant lethal allele of a gene expressed at the larval stage, so all of the offspring will be dead early. The Wolbachia bacteria instead impede replication of the virus in the mosquito and in that way limit the spread of the pathogen (111,112).

It is important to educate people and especially advise pregnant women to avoid unnecessary travelling to endemic regions of Zika virus to prevent mosquito bites and ZIKV infections. For individual protection it is good to use long pants, light-coloured clothing and insect repellents. Treating clothes, bed nets, air conditioning systems and window screens with permethrin, a mosquito repellent, has shown to be effective in reducing arthropod bites (100,113,114). When sexual transmission is concerned, CDC has made guidelines how to reduce the spread of the virus and above all to prevent infections of pregnant women. Men living or travelling in countries of autochthonous ZIKV transmission should use condom during sexual intercourse with their pregnant partner. Barrier contraceptives are also recommended if the male partner has visited endemic area and is concerned about sexual transmission of ZIKV, even if the female partner is not pregnant. Authorities have also given a guidance to use a condom for 28 days after returning from an endemic area, if traveller is asymptomatic, and for 6 months if he or she is having symptoms (115,116) .

Vaccines against yellow fever, Japanese encephalitis, tick-borne encephalitis and dengue viruses exist but there is no clinically approved ZIKV vaccine. Same strategies that appeared to be potential in vaccination against the other flaviviruses are used in ZIKV vaccine development: live attenuated and purified inactivated viruses, glycoprotein

subunits and DNA vaccines (94,117). Structural glycoproteins E and prM and virus-like particles containing both structural and non-structural proteins have provoked antibody production in mice (118-122). It has been estimated that one single vaccine could work effectively against all Zika strains because there is relatively little genetic variation between different viral lineages. It has been showed in murine model that infection with African lineage produces antibodies that are able to effectively neutralize ZIKV of Asian lineage, too(123). Because dengue virus immunity is common in ZIKV endemic areas, the interaction between dengue antibodies and the upcoming ZIKV vaccine need to be studied. It has been hypothesized that the cross-reacting antibodies either boost or reduce the immunity against ZIKV. There is also a possibility that when using live attenuated viruses, the dengue antibodies can directly neutralize the viruses of the vaccine (101).

3 Aims of the study

To investigate the underlying reasons why ZIKV, previously causing only mild, self-limiting symptoms, has recently caused a vast epidemic with severe neurologic consequences, we set the following aims:

- To compare the infectivity and replication efficiency of the recent foetal brain isolate to two other genetically different ZIKV epidemic strains of Asian lineage, as well as to the ancient prototypic African strain, in 10 different human cell lines essential in ZIKV pathogenesis
- To compare the replication efficiency of these strains in cell lines originating from the mosquito vector species, and
- To investigate the first line antiviral responses in mammalian cells.

4 Materials and methods

4.1 Cells and viruses

We chose 10 different human and three nonhuman cell lines to be included in the study (Table 1). Human cells were sustained in Eagle's Minimal Essential Media (MEM), Dulbecco's Modified Eagle Medium, or PRMI 1640 supplemented with glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin and 10 % FBS at 37 °C with 5% CO_2. Mosquito cells were maintained in Leibovitz's L-15 medium supplemented with glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin and 5% FBS at room temperature. During infections 2% FBS was added in the media.

Table 1. Human and mosquito cells used in the study

Cell line	Anatomic origin	Source	Culture media
Human			
HaCaT	Keratinocyte cell line from adult human skin	Kindly provided by Prof. Dr. Petra Boukamp ans Prof. Dr. Norbert E. Fusenig, DKFZ, Heidelberg, Germany	D-MEM
HFSF	Human foreskin fibroblast	Kindly provided by Dr. Magdalena Eisinger, Memorial Sloan-	D-MEM

		Kettering Cancer Center, New York, NY, USA	
MRC-5	Fetal lung fibroblast	ATCC®CCL-171™	MEM
SK-UT-1	Uterus epithelial grade III mesodermal tumor (mixed) consistent with leiomyosarcoma	ATCC®HTB-114 ™	MEM
JAR	Placenta epithelial choriocarcinoma	ATCC®HTB-144 ™	RPMI 1640
HUVEC	Umbilical vein/vascular endothelium	ATCC®CRL1730™	EBM MV
HMEC-1	Dermal microvascular endothelium, newborn	ATCC®CRL-3243 ™	EBM MV
A498	Kidney epithelial carcinoma	ATCC®HTB-44 ™	MEM
H-2	Glioblastoma cell line from a rapidly expanding local brain tumor	(124)	MEM
H-4	Brain epithelial neuroglioma	ATCC®HTB-148 ™	D-MEM
Nonhuman			
mosquito			

AE	*Aedes aegypti*	Kindly provided by X. de Lamballerie	L-15 supplemented with tryptose-phosphate broth
C6/36	*Aedes albopictus*		L-15
AP-61	*Aedes pseudoscutellaris*		L-15 supplemented with tryptose-phosphate broth

ZIKV FB-GWUH-2016 is a strain (Asian lineage) isolated from foetal brain tissue in SK-N-SH (human neuroblastoma) cells (10). MR766, an African strain, was provided by Dr. Jonas Schmidt-Chanasit (Berhard-Nocht-Institut für Topenmedizin, Hamburg, Germany). MRS_OPY_Martinique_PaRi_2015 (clinical isolate from Martinique 2015; MRS OPY) and H/PF/2013 (clinical isolate from French Polynesia 2013; H/PF) were acquired from EVA (European Virus Achive, Marseille, France) and both isolated from sera of symptomatic adult patients. MRS OPY and H/PF were transported freeze-dried and later propagated once in VeroE6 cells, resulting in total number of passages 6 and 2, respectively. MR766 was propagated twice in VeroE6 cells for this study but its previous number of passages is unknown. All viral titers were measured by end point method in VeroE6 cells.

For infections, cells were transferred into T25 flasks and grown to 80% confluency. Infections were done in BSL-3 laboratory at +37° C (human cell lines) or at room temperature (mosquito cell lines) with incubation time of 1h. MOI (multiplicity of infection) was approximately 0,1. After infection the cells were washed once with PBS,

the cell culture media was added and the cells were incubated for three days at 37 °C (human cell lines) or at room temperature (mosquito cell lines).

4.2 RNA extraction and quantitative RT-qPCR

Amount of ZIKV RNA was measured from the culture supernatant at 0 and 3 days post infection (d.p.i.) by quantitative RT-PCR. RNA was extracted by using Qiagen Viral RNA Mini Kit according to manufacturers' instructions. RT-qPCR was performed with TaqMan® Fast Virus 1-Step Master Mix and Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Primers targeted to ZIKV NS5 protein and TaqMan probe with VIC-dye were used as described previously (10).

Cellular RNA was extracted using RNeasy Mini Kit (Qiagen) according to manufacturers' instructions at 0 and 3 d.p.i. We used qScript One-Step SYBR Green RT-qPCR (Quantabio, Beverly, MA, USA) to measure the expression of cellular MxA and RNA polymerase II from 50ng of total cellular RNA. RNA polymerase II was used as a housekeeping gene. Primers used for MxA were forward: 5'-AGTATGGTGTCGACATACCGGA-3` and reverse: 5`-AGTATGGTGTCGACATACCGGA-3. For RNA-polymerase II primers were forward: 5`-GCACCACGTCCAATGACAT-3` and reverse: 5`-GTGCGGCTGTTCCATAA-3`. Water was used as a negative control and each sample was analyzed in duplicate.

4.3 Virus titration

Pre- and post-infectious viral loads, expressed as logTCID50 values were measured by end point titration method. Briefly, VeroE6 cells were seeded to 96-well plates and infected with quadruples of each virus dilution from three independent trials. Cytopathic

effect was observed and logTCID50 values were calculated as more precisely described in an article by Reed and Muench (125).

4.4 Immunofluorescence assay (IFA)

IFA slides were prepared of cell suspension collected 3 d.p.i. and fixed with ice-cold 100% acetone. Serum of a ZIKV IgG positive patient, diluted to 1:80 was used as a primary antibody in staining the ZIKV antigens. Fluorescein (FITC)-conjugated goat anti-human IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA) was utilized as a fluorescent secondary antibody. Pictures were taken with Olympus BX51 Fluorescence Microscope and processed with Olympus DP Controller software.

4.5 Statistical analysis

All statistical analyses were done with student`s t-test and P-value <0.05 was considered statistically significant.

5 Results and discussion

5.1 Human cell lines

We found differences in the replication properties of the different ZIKV strains in many of the cell lines of human origin (Fig. 4). FB-GWUH was able to replicate in all of the studied cell lines and showed higher replication efficiency, and infectious virus production, in uterus epithelial cells (SK-UT-1), glial cell line H4 (from neuroglioma), dermal microvascular cells of a newborn (HMEC1) and kidney cells (A498), when compared to the other two Asian strains (Fig. 5). In foetal lung fibroblasts (MRC5), glial cells (H2), vascular endothelial cells of the umbilical vein (HUVEC) and adult keratinocytes (HaCaT), FB-GWUH had the highest viral RNA load and the production

of infectious viral particles was more effective when compared to MRS OPY and MR766 (Fig. 4 and Fig. 5). In these cell lines, there was no statistical difference in the production of infectious viral particles between FB-GWUH and H/PF.

In uterus (SK-UT-1) and skin (HaCaT) cells, FB-GWUH and MR766 had higher viral RNA load 3 d.p.i. than MRS OPY and H/PF, but the production of infectious viruses was highest in H/PF-infected HaCaT-cells (Fig. 4 and Fig. 5). FB-GWUH and MR766 infected also H2 and H4 cell lines most efficiently, when measured by viral RNA load (Fig. 4). H2 and H4 are both cell lines derived from tumours of the central nervous system. H2 is a cell line originating from a glioblastoma, consisting mostly of astrocytes. ZIKV has been shown to infect astrocytes and, according to a previous study, an African strain ArB41644 was more efficient than H/PF (84,124). In astrocytes, there is an overexpression of the AXL-receptor, which is a possible candidate for the entry of ZIKV (83,126,127). H4, in which cells the replication was lower, is a cell line originating from a neuroglioma, which can be derived from astrocytes, oligodendrocytes or ependymal cells. All of the studied viruses caused cytopathic effect (CPE) in H2, but in H4, only FB-GWUH was able to induce CPE. The infections of these two neuronal cell lines could not be visualized by antigen production (IFA), except for FB-GWUH infected H2 cells (Fig. 6). Based on these differences in effectiveness of ZIKV infection in these two cell lines, both derived from brain tumours, ZIKV may have cell preferences or specificities even inside the central nervous system.

Endothelial cells of the umbilical vein (HUVEC) and kidney cells (A498) were susceptible to all of the ZIKV strains. CPE was observed and huge viral loads were

measured by RT-PCR (Fig. 4). The infectivity of viruses from these cell lines at 3 d.p.i. was also high (Fig. 5). In IFA, the ZIKV antigen positivity was strong in HUVEC, and to a lesser extent in A498, for all four virus strains (Fig. 6). Placental cells (JAR) were infected by FB-GWUH, MR766 and H/PF (Fig. 4). Cells were antigen positive for all of the viruses, reactions for FB-GWUH and MR766 were strongest compared to all of the studied cell lines (Fig. 6). To support the strong infectivity in JAR and HUVEC cells, CPE was seen in these cell lines. These results are supported by previous studies. El Costa and others showed that ZIKV is able to infect and damage various cell lines from placenta, maternal decidua and umbilical cord during the first trimester of pregnancy. Trophoblasts, mesenchymal/fibroblast-like cells and placental macrophages in addition to mesenchymal stem cells of umbilical cord were all susceptible to ZIKV infection (128). Placental cell line JEG3 also showed high viral loads, expression of ZIKV proteins and CPE in a study by Chan *et al*. They also compared MR766 to a clinical isolate from Puerto Rico (PRVABC59) and reported higher viral loads for the African strain in JEG3 at 3 d.p.i. In our study, the epidemic strain from French Polynesia H/PF replicated effectively in JAR cells with infectious virus production comparable to that of MR766 and FB-GWUH (Fig. 5). Chan *et al*. also reported considerable viral loads but no CPE in renal cell line (HEK). This is consistent with our results in A498. It might be that the kidneys and other cells of the genitourinary tract have a role in sexual transmission and are sites of persistent ZIKV replication (129).

In dermal microvascular endothelial cells of newborn (HMEC-1) only FB-GWUH could produce statistically significant difference in RNA loads and the infectious virus production was highest in FB-GWUH infected cells (Fig. 4 and Fig. 5). Two fibroblast

cell lines were included in this study, HFSF and MRC-5. HFSF is a foreskin fibroblast and MRC-5 is from foetal lung. MRC-5 was efficiently infected by all of the viruses and HFSF by all but MRS OPY. FB-GWUH had the highest viral loads and infectious virus production in these cell lines (Fig. 4 and Fig. 5). Immunofluorescent reaction was stronger in MRC-5 (Fig. 6). No CPE was observed in these cell lines.

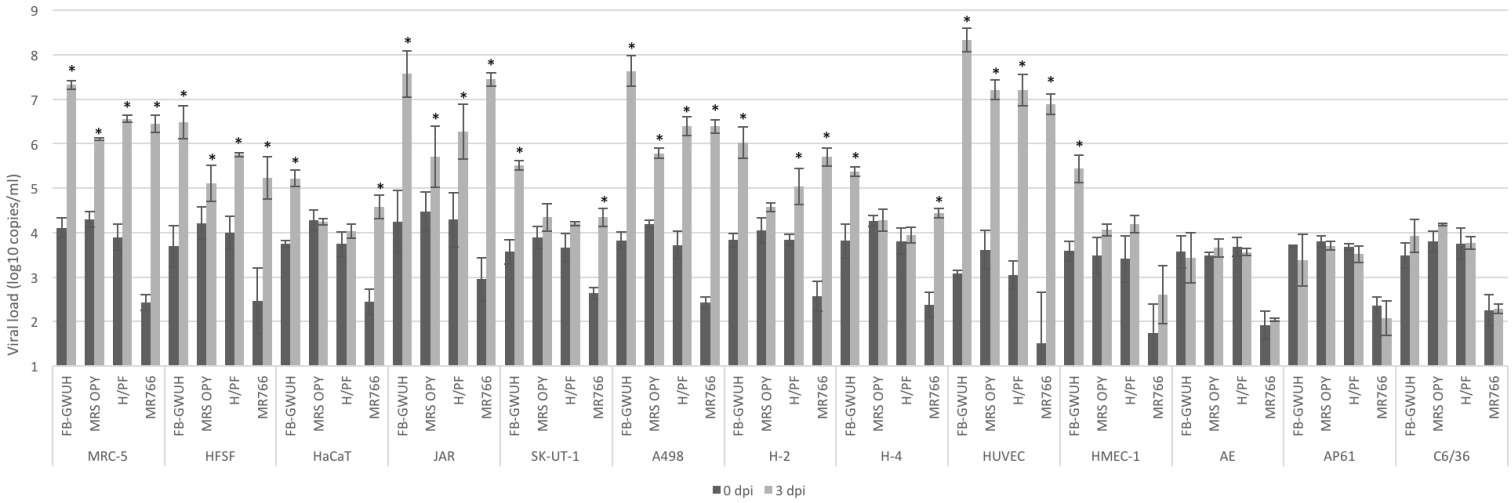

Figure 4. ZIKV RNA load in cell supernatants 0 and 3 d.p.i. The presented values are calculations from log-transformed viral loads from three independent experiments with the exception of the SK-UT-1 and mosquito cell lines, which were from two experiments. *P-value <0,05 compared to day 0.

5.2 MxA

In order to gain an insight into the antiviral responses in the studied mammalian cell lines, we measured the antiviral MxA mRNA in the infected cells. We also wanted to find out if the differences in its production would correlate with the variabilities in the virus infectivity in different cell lines. MxA is a cytoplasmic GTPase, which has broad antiviral activity against various viruses (130). It is induced by α- and β-interferons, which are type I interferons. MxA has an ability to transiently bind many proteins of the cytoskeleton (131). It has been shown that Zika virus is able to inhibit the production of type I interferons and transcription of interferon-stimulated genes. Viral proteins NS1, NS4A

and NS5 are able to suppress the signalling pathway leading to induction of type I interferons (132). NS5 also binds STAT2 stimulating its proteasomal degradation and in this way inhibits the reading of interferon-stimulated genes (133).

Generally, the measured MxA levels were quite low in our study, which is in accordance with the previous finding of ZIKV-induced inhibition of interferon I signaling (Fig. 7). The amount of MxA expression did not correlate with the effectiveness of ZIKV replication in the cells; high viral RNA amounts were observed in both the cell lines expressing high MxA levels and the cells with no MxA expression. There was no considerable MxA expression in uterus, umbilical cord, placenta, newborn microvascular endothelium, brain or skin (Fig. 7). The highest MxA levels were detected in renal cells (A498). Of the two fibroblast cell lines, the MxA expression was higher in MRC-5. To understand the role of ZIKV infection in these observed MxA levels and the function of other cellular antiviral responses during ZIKV infection needs further studies.

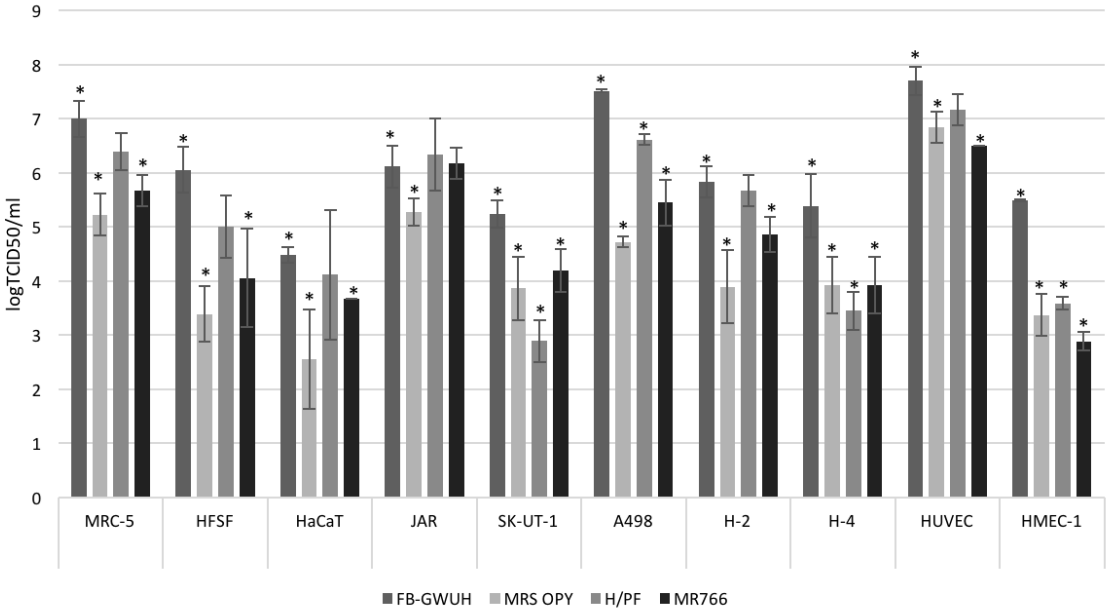

Figure 5. Infectious virus production in 10 human cell lines 3 d.p.i., expressed as logTCID50/ml.

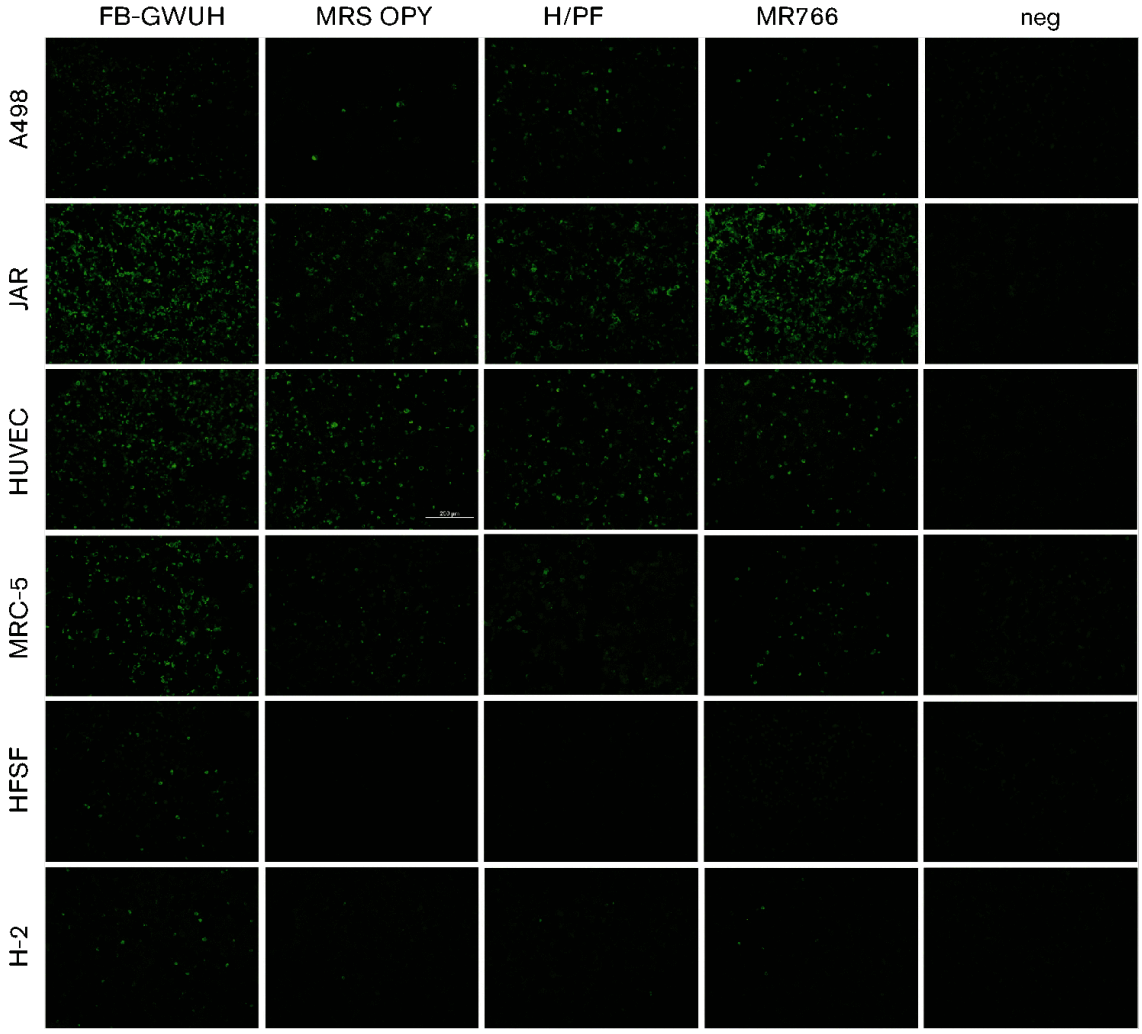

Figure 6. ZIKV antigen detection by immunofluorescence staining. Scale bar 200µm.

5.3 Mosquito cell lines

We tested the replication capability of the ZIKV strains also in three mosquito cell lines: *Aedes aegypti*, (AE), the main vector of ZIKV*; Aedes albopictus* (C6/36), a mosquito cell line widely used in isolations of mosquito-borne viruses and *Aedes pseudocutellaris* (AP-61). The viral RNA loads were measured 3 d.p.i similarly as for human cell lines. The observed viral loads were considerably lower than in human cell lines, but all of the ZIKV strains could replicate in these cells (Fig. 4). Surprisingly, FB-GWUH and MR766 had lowest viral loads in all mosquito cell lines; especially RNA levels of MR766 remained

unexpectedly low. The viral loads were highest in C6/36, a cell line known to have a deficient antiviral RNA interference response (134). These results support a previous study that reported the epidemic strain from Puerto Rico (PRVABC59) to replicate more efficiently than MR766 and another African strain in C6/36 and AE cells. In the same study MR766 was shown to have highest viral loads in hepatocyte-derived carcinoma cells (HUH7) (51,134). The reason for low replication numbers in mosquito cells lines might be that because of the mosquito being a vector, the virus does not have to actually infect the cells and produce huge loads of viral RNA in order to be spread to blood and in that way to a next vector and next host. The virus maintains a moderate replication, enough to be transmitted to the next host through the saliva of the mosquito.

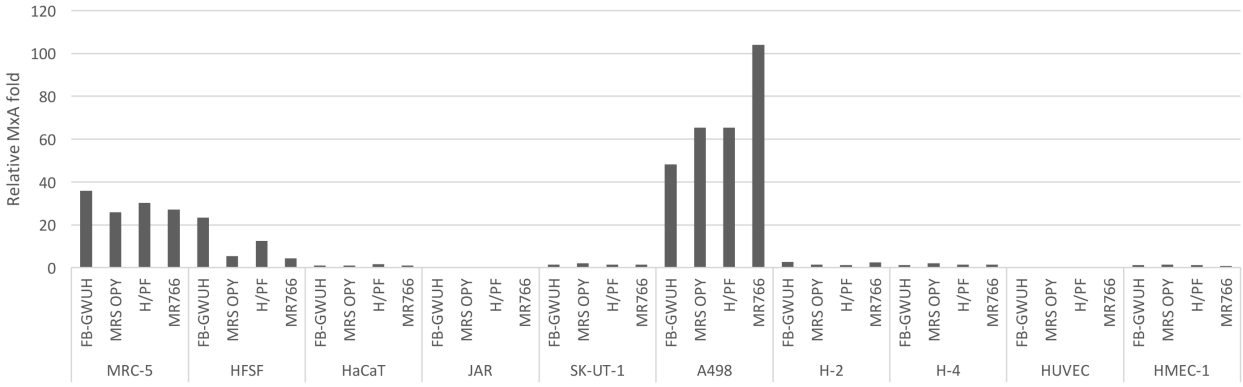

Figure 7. The relative induction of MxA transcription.

In conclusion, we found differences in infectivity between the different ZIKV strains of Asian lineages in cell lines originating from the central nervous system. The highest infectious virus production of FB-GWUH in brain cells may support the hypothesis of new adaptations developed in the genome of the virus during the latest outbreak, thus making the virus more capable to infect the cells of the nervous system. Genetically compared, there are 14 polymorphic amino acid sites between the three Asian strains used

in this study, of which 9 are specific to FB-GWUH so they do not exist in the genome of MRS OPY or H/PF. The closest relatives of FB-GWUH are strains isolated from Guatemalan patients, to which GWUH has 8 amino acid changes. One of those is located in NS5, protein important in viral RNA production, which could in part explain the generally more efficient replication of GWUH in our study. Another alteration in the amino acid chain is in protein NS2B, which also has a crucial role in replication. GWUH also has a variant amino acid in protein E that is needed in viral entry. This change may affect the receptor binding and cell tropism of the virus and in this way could be explaining for example the neuroinvasiveness of the foetal brain isolate.

FB-GWUH has 10 amino acid differences to H/PF and 13 to MRS OPY. H/PF and MRS OPY differ in 5 amino acids from each other. Because of the small number of genetic variation, only one or some of these genetic differences are likely to explain the variation in cellular infectivity between the strains. To estimate the role of specific amino acid substitutions between the prototypic strain MR766 and the Asian strains is quite challenging because there are more than 100 of them.

Still, one interesting variation, taking into account the similarities in replication between FB-GWUH and MR766 that we observed, is that FB-GWUH and MR766 have both threonine in site 2679 (NS5), whereas MRS OPY and H/PF have alanine, which brings FB-GWUH towards the African lineage. However, further studies using reverse genetics are needed to explain the roles of single amino acid substitutions. The possibly common adaptations that have arisen in the genome of FB-GWUH and MR766 may be due to persistent infection of the fetoplacental system, repeated infections of the mammalian

cells and extensive number of passages for MR766. Infectious virus production and viral RNA loads were high in all of the studied cell lines for FB-GWUH, which may indicate that this strain has as a whole become more effective in infecting human cells.

The major limitation of our study is the usage of immortalized cell lines instead of organoids, primary cell, organ or animal models, which better mimic the real tissue structure. However, cell culture is a simple and cost-effective manner to screen for variation in growth properties of the viruses and our study revealed considerable differences in the replication capabilities between the ZIKV strains. This study forms a good basis for further more detailed studies to examine the replication kinetics of Zika viruses and to genetic research to explain the reasons behind the observed differences.

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